

Seasonal variations of the microflora of wedge clam *Donax trunculus* (Linnaeus, 1758) from the region of Arkutino (Bulgarian Black Sea aquatory)

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Abstract

The main goal of the present study was to investigate the impact of the state of the environment on the microbiota of the wedge clam *Donax trunculus* (Linnaeus, 1758) from the region of Arkutino (Bulgarian Black Sea aquatory). The species *Enterococcus hirae* was isolated during the summer (from May to August). The species *P. mendocina* prefers the warmer months and the species *P. alcaligenes* the colder ones. The temperature followed a course of decrease during the period September 2020 to January 2021, followed by a slow increase from February 2021. Comparing May 2020 with May 2021, it became evident that in 2021 the temperature was 1.5 °C lower. We can say that the number of the species *P. alcaligenes* was twice as high in May 2021. It is likely that this species preferred lower optimum temperatures and constant other parameters. For the species *Enterococcus hirae* such dependence was not observed - the number remained constant in May, but with increasing temperature the number of microorganisms decreased during the summer months. The species seemed to preferably develop at pH 7.78. The species *A. gyllenbergii* preferably grows at temperatures between 20.3–25.7 °C and the optimal temperature was 25.7 °C. For *C. farmeri* the optimum conditions were temperature 26.2 °C and pH 7.3. The species *E. vulneris* was probably related not only to the increase in water temperature, but also to the anthropogenic factor, as it was found only in July.

Keywords

Black sea, *Donax trunculus*, microbial identification, pathogens

Introduction

Among marine bivalve species, the wedge clam *Donax trunculus* (Linnaeus, 1758) has one of the highest yields in the world. The habitat of this species is close to open sandy beaches, where it forms thick beds. It is found along the Atlantic-Mediterranean coast with the highest density at a depth from 0 to 3 m (Gaspar et al. 2002). The highest population density is reached at the surf. The species was found in the Mediterranean and the Black Sea from Senegal to the North Atlantic coast of France (Deval 2009). According to The State of World Fisheries and Aquaculture (2016) the annual production of *Donax* in Europe reaches 970–1353 tons. Both *Donax* species - *D. trunculus* and *D. variegatus* can be found along the Black Sea coast, but the population of *D. trunculus* (Fernández-Pérez et al. 2017) has a higher density. *D. trunculus* is commercially important in many countries like France, Italy, Turkey, Portugal and Spain as a food resource. In Galicia, *D. trunculus* is the bivalve with rising commercial value in the markets during recent years (42.37 €/kg in the year 2017), and its value has increased with the consequent increase in its fishing pressure (Fernández-Pérez et al. 2017). In Recent years have registered an increase in sites on the southern Black Sea coast where *D. trunculus* has been extracted; it is then mainly exported abroad on account of its high prices. Due to excessive collecting in some spots (including Bulgaria), the wild stocks are drastically depleted. Shellfish are exposed to diseases caused by various bacteria, which can also cause a mass extinction of species along the coast. It was detected, that the cause of outbreaks of diseases in bivalves is related to conditional pathogens, i.e. free-living pathogenic bacteria which, under favorable conditions, can cause diseases. This poses a serious risk to humans as consumers of clam species. Pathogenic bacteria can enter into the clams from seawater, from the microalgae they feed on, and as a result of anthropogenic pollution of the environment. The species *D. trunculus* is most often used as a bioindicator, as it is very sensitive to changes in the environment (Signorelli and Raven 2018). We have found no scientific publications on the microbiological status of wedge clams *D. trunculus* from the Bulgarian Black Sea aquatory and the impact of the state of the environment on it.

The main goal of the present study was to investigate the impact of the state of the marine environment on the microbiota of the wedge clams *D. trunculus* from the region of Arkutino (Bulgarian Black Sea aquatory).

Materials and methods

Place and duration of the study

The samples were collected from the region of Arkutino (exact field coordinates 42.3341 N, 27.7317 E: Datum WGS 84) from May 2019 until May 2021. The laboratory studies were conducted at the Department of Biology, University of Shumen, Bulgaria.

Sample collection

The wedge clams were harvested from the Bulgarian Black Sea aquatory. After collection of the three subsamples (each of about 1 kg), they were refrigerated (4 °C) and transported to the laboratory for further immediate analysis, without freezing the specimens.

In this study, we examined wedge clams of similar size, weight, and shape to ensure maximal uniformity in the applied methods (Duquesne et al. 2004). The average length of mussels used in the study was 2.2 ± 0.43 cm.

Physico-chemical analysis of the inhabited sea waters

During the mussel sampling, we measured in situ the temperature, total salinity (by using YSI Model 33 salinity meter), and pH (by using ATC Piccolo HI1280 pH-meter).

Microbiological analysis

Three subsamples (each of about 1 kg of wedge clams) were used for the microbiological analyses. The clams were scrubbed free of dirt, washed in hypochlorite solution (20 mg l⁻¹), rinsed with sterile distilled water, and shucked with a sterile knife. Tissue liquor samples (about 100 g) were homogenized (Maffei et al. 2009).

Fecal coliforms (FC) were enumerated through five tubes per dilution most probable number (MPN) series (Ignatova-Ivanova et. al. 2018). After 3 h at 37 °C plus 21 h at 44 °C, gas positive tubes were recorded for FC. From each FC gas positive tubes, 0.1 ml were transferred in tubes with 10 ml of Tryptone Water (Oxoid, Basingstoke, UK) and then incubated for 24 h at 44 °C. *E. coli* was enumerated by MacConkey agar (Merck, Darmstadt, Germany). The plates were incubated aerobically at 35–37 °C for 18–24 hours. *E. coli* grows matte dark pink to tile red, surrounded by an opaque area due to the precipitation of bile salts in this environment. *Pseudomonas* sp. was enumerated by Cetrimide Agar (Merck KGaA, 64271 Darmstadt, Germany).

Microbial identification databases for the “Biolog” systems

The microbial identification was performed by the Biolog Microbial Identification System (VIO45101AM). The isolated strains were screened on BL4021502 Tryptic Soy Agar (TCA), cultured for 24 hours at 37 °C, and then subjected to Gen III plaque identification to identify Gram-positive and Gram-negative aerobic bacteria. The microscopic pictures were made using stereomicroscope OPTIKA (Italy) with a Dino-Eye, Eyepiece camera with 5 megapixels. The photographs were performed by using a Canon EOS 60D camera. The GEN III MicroPlate test panel provides a standardized micromethod using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria. Biolog’s Microbial Identification Systems software (e.g. OmniLog Data Collection) is used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate. The BIOLOGIST system allows to quickly and accurately identify more than 2900 species of aerobic and anaerobic bac-

Table 1. The physico-chemical parameters of the marine water.

Region	Date	Depth	Temperature	pH	Salinity	Dissolved $_{\text{O}_2}$
		M	[° C]	[pH]	[ppt]	[mg/l]
Arkutino	05.2020	2 to 4	24.4	7.78	12.2	7.9
Arkutino	06.2020	2 to 4	25.5	7.32	13.5	7.7
Arkutino	07.2020	2 to 4	27.2	8.26	13.5	7.8
Arkutino	08.2020	2 to 4	27.7	8.36	11.2	8.07
Arkutino	09.2020	2 to 4	26.2	8.20	11.2	8.1
Arkutino	10.2020	2 to 4	25.7	8.13	11.2	8.1
Arkutino	11.2020	2 to 4	22.5	7.2	12.78	6.9
Arkutino	12.2020	2 to 4	19.9	6.5	12.69	6.8
Arkutino	01.2021	2 to 4	19.8	6.5	12.71	6.9
Arkutino	02.2021	2 to 4	20.3	6.9	12.78	7.5
Arkutino	03.2021	2 to 4	22.5	7.3	12.99	7.7
Arkutino	04.2021	2 to 4	23.1	7.4	13.1	7.7
Arkutino	05.2021	2 to 4	22.9	7.70	13.3	7.6

teria, yeasts, and fungi. Biolog's advanced phenotypic technology provides valuable information on the properties of the strains, in addition to species-level identification. Biolog's carbon technology identifies the environment and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" of discrete test reactions performed in a 96-well microplate. The culture suspensions are tested with a panel of pre-selected assays, then incubated, read and compared with extensive databases. <https://www.biolog.com/products-portfolio-overview/microbial-identification/>

Results

We conducted a physicochemical analysis of the sea waters. The results are summarized in Table 1. The data represents results from the measurement of 4 basic physicochemical parameters.

The dynamics of changes in the physicochemical parameter values are shown in Fig. 1.

From the data presented in Fig. 1, it is visible that the temperature follows a course of decrease from September 2020 to January 2021, followed by a slow increase from February. If we compare May 2020 with May 2021, is evident that the temperature in 2021 was 1.5 °C colder (Fig 1a). Comparing the results in Fig. 1 we can say that the number of the species *P. alcaligenes* was twice as high in May 2021. It is likely that this species preferred to grow at lower optimum temperatures and constant other parameters. For the species, *Enterococcus hirae* such dependence was not observed - the number of this strain remained constant in May, but with increasing temperature, the number of microorganisms decreased during the summer months. Only *Enterococcus* clarify preferably developed at pH 7.78 (Fig. 1b). For the species *A. gyllenbergsii*, we can say that it preferably grew at temperatures between 20.3–25.7°C, and the optimal temperature was 25.7 °C. For *C. farmeri* the optimum temperature was 26.2 °C and the pH 7.3. The species *E. vulneris* was probably related not only to the increase in water temperature but also to the anthropogenic factor, as it was found only in July. The

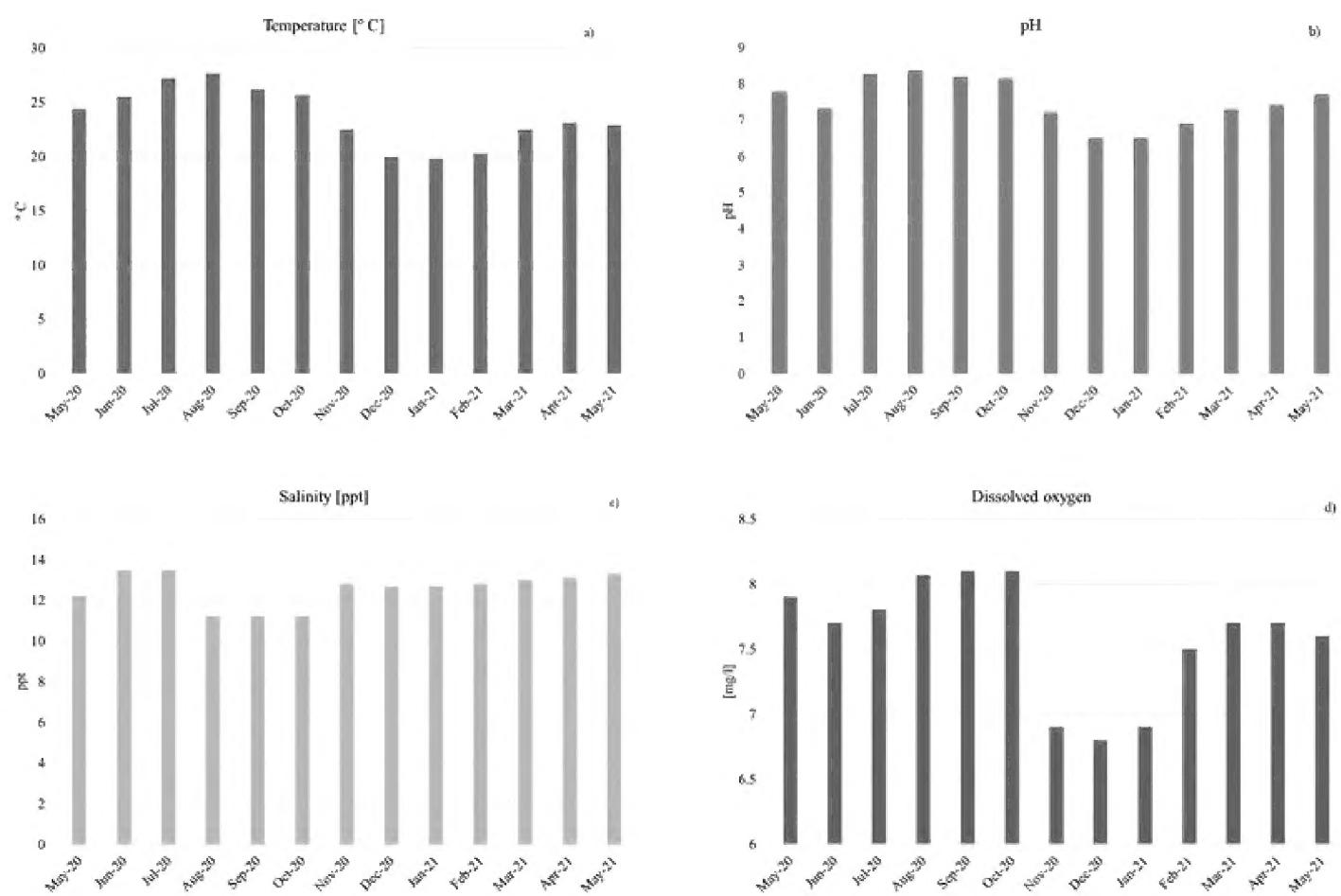


Figure 1. Dynamics in the change of physicochemical parameters of marine water.

Table 2. The number of bacterial cells in 1 ml on the different media.

Region/clam species	Pseudomonas agar	Cetrimid agar	Chromokult agar	MacConkey agar	Strain BIOLOG
Arkutino 17.05.2020/ <i>D. trunculus</i>	3.8×10^4			8.4×10^4	<i>Enterococcus hirae</i> <i>Pseudomonas mendocina</i>
Arkutino 20.06.2020/ <i>D. trunculus</i>				5.3×10^4	<i>Enterococcus hirae</i>
Arkutino 25.07.2020/ <i>D. trunculus</i>				1.6×10^7	<i>Escherichia vulneris</i>
Arkutino/ 25.08.20 <i>D. trunculus</i>				5.8×10^4	<i>Enterococcus hirae</i>
Arkutino/ 02.09.2020 <i>D. trunculus</i>				3.4×10^4	<i>Citrobacter farmeri</i>
Arkutino 17.10.2020/ <i>D. trunculus</i>				9.2×10^6	<i>Acinetobacter gyllenbergsii</i>
Arkutino 18.11.2020/ <i>D. trunculus</i>	8.9×10^6				<i>Pseudomonas alcaligenes</i>
Arkutino 18.12.2020/ <i>D. trunculus</i>	1.34×10^7				<i>P. Pseudomonas alcaligenes</i>
Arkutino 18.01.2021/ <i>D. trunculus</i>	3.4×10^4				<i>P. Pseudomonas alcaligenes</i>
Arkutino 18.02.2021/ <i>D. trunculus</i>				4.4×10^4	<i>Acinetobacter gyllenbergsii</i>
Arkutino 18.03.2021/ <i>D. trunculus</i>				8.4×10^5	<i>Acinetobacter gyllenbergsii</i>
Arkutino 18.04.2021/ <i>D. trunculus</i>	3.2×10^4				<i>P. mendocina</i>
Arkutino 17.05.2021/ <i>D. trunculus</i>	7.4×10^4			7.3×10^3	<i>Pseudomonas mendocina</i> <i>Enterococcus hirae</i> -

lowest amount of dissolved oxygen was from November to January, when the species *P. alcaligenes* preferably develops (Fig. 1c).

The microorganisms isolated from *D. trunculus* were determined by the Biolog Microbial Identification System (Biolog VIO45101AM) to species level. After 24 h of cultivation on different media, the number of cells in 1 ml were obtained - data represented in Table 2 and Fig. 2.

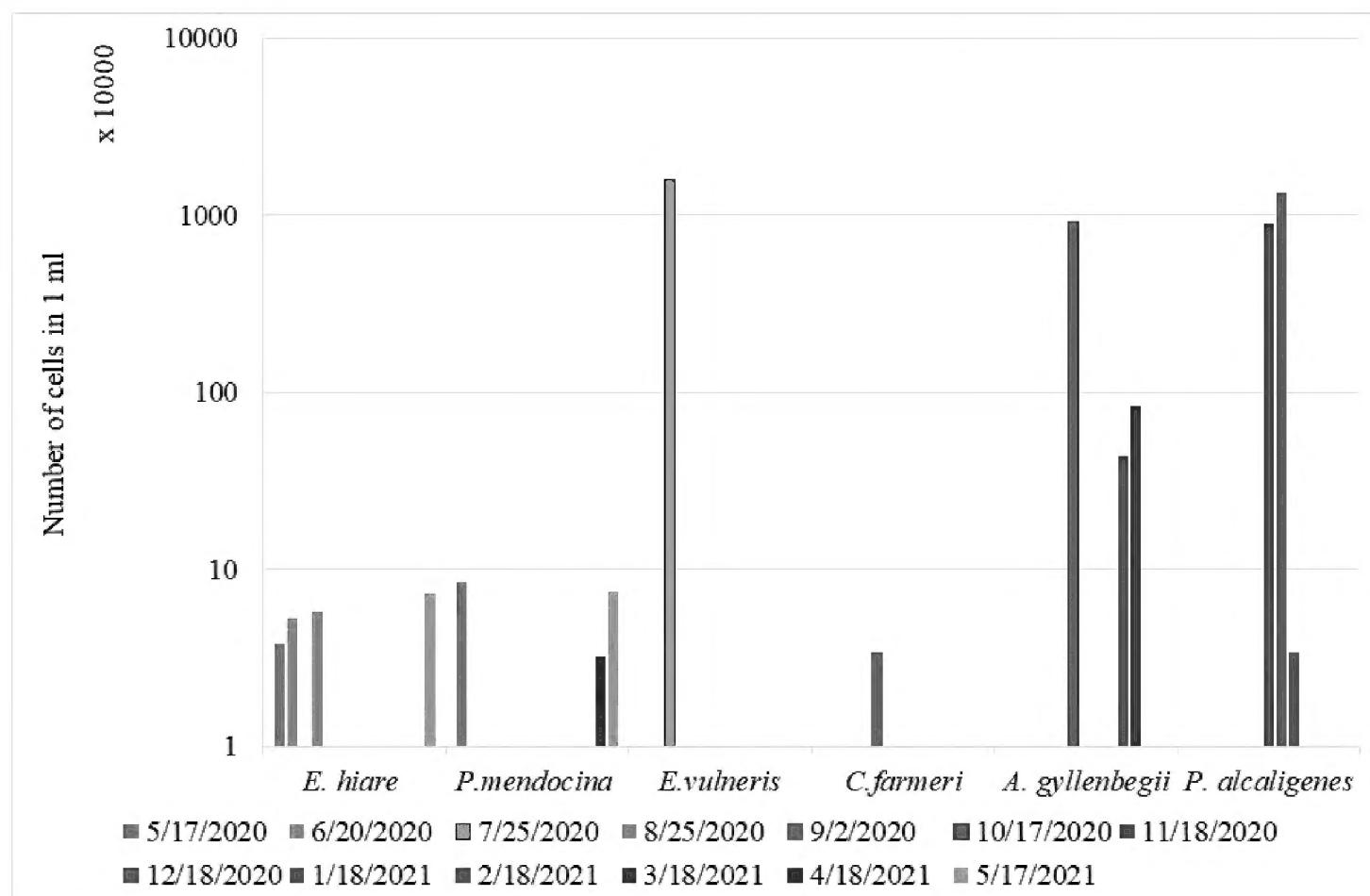


Figure 2. Dynamics of microorganism species, isolated from wedge clams for 2020–2021 period.

Fecal coliforms (FC) were represented by the species *Escherichia vulneris*, which was isolated only in July in a significant number of 1.6×10^7 (Table 2; Fig. 2). The species *Enterococcus hirae* was isolated during the period from May to August (Table 2; Fig. 2). From genus, *Pseudomonas* (Table 2; Fig. 2) were detected *P. mendocina* (isolated in April and May) and *P. alcaligenes* (isolated in November, December and January). The species *Citrobacter farmeri* was isolated in September, and the species *Acinetobacter gyllenbergsii* in February–March. The distribution dynamics of different types of microorganisms isolated from the wedge clam *D. trunculus* are represented in Fig. 2. Fig. 1 shows that the species *Pseudomonas* preferably developed in the cooler months, although there was a variation within the genus, as well. The species *P. mendocina* preferred the warmer months, while the species *P. alcaligenes* preferred the colder ones.

Discussion

The microbiota found in marine organisms, and mussels in particular, can be considered in two respects - the so-called 'resident' microbiota, which is stable and unaffected by the environment, and the 'transitional', which depends on the environmental conditions. Specifically, our studies show that fecal coliforms represented by *E. vulneris*, which was isolated only in July, *Citrobacter farmeri*, which was isolated only in September, most likely belong to the transitional species. On the opposite, the species of *Pseudomonas* sp., *Enterococcus* sp. and *Acinetobacter gyllenbergsii* are resident for the in-

vestigated mussels. Our results demonstrate an increase in the quantity of the coliforms in the region of Arkutino in July, when the quantity of the fecal coliforms is 190 times over the norms prescribed in Ordinance No. 4/20.10.2000 for the quality of fisheries water and the breeding of shellfish (the number of fecal coliforms in the inter-shell content should be less than 300 NVB). This can be harmful to human health following the consumption of mussels. Jorquera et al. (2001) suggested that the bivalve mollusks only present "transition" microbiota. In general, the resident microbiota performs various functions in mussels - it serves as food, a source of vitamins and growth factors, plays also a role in the defense mechanisms to prevent the colonization of bacterial pathogens or eliminate toxic substances (Prieur et al. 1990; Seguineau et al. 1996). On the other hand, the microbiota can also enter the mussels as a result of environmental pollution. According to data from the literature, many microorganisms belonging to different species – such as *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Photobacterium*, *Moraxella*, *Aeromonas*, *Micrococcus*, and *Bacillus* (which are part of the bacterial population in the aquatic environment) may enter the mussels as a result of the diet by filtration. The first study analyzing the normal microbiota of a bivalve species was performed by Colwell and Liston (1960) with the Pacific oyster (*Crassostrea gigas*). The authors found a high proportion of Gram-negative bacteria (> 80%), with a predominance of the genera *Pseudomonas* and *Vibrio*, but also of *Flavobacterium* and *Achromobacter*. In general, the detected bacteria can be considered as typical psychrophilic marine bacteria, physiologically adapted to survive within the bivalve. In the literature, studies analyzing the diversity, distribution, and density of marine bacteria associated with bivalve mollusks are scarce. In fact, most studies have focused on characterizing bacteria with pathogenic potential for mollusks, or specifically for mussels. Thus, the genus *Vibrio* is widely studied, as it is known as one of the most important bacterial genera affecting the culture of bivalves. For example, *V. tapetis* has received special attention since it caused Brown Ring Disease (BRD), the bacterial etiology of which is described in adult clams. In addition, the disease caused by it is considered one of the main limiting factors for the culture of Manila clams (*Venerupis philippinarum*) (Europe Borrego et al. 1996), and was also detected in cultured clams in Korea (Park et al. 2006). Environmental parameters, such as variations in temperature and salinity, can affect the diversity of microorganisms and the environment as a physiological state of bivalves and its susceptibility to bacterial infections (Arias et al. 1999; Pujalte et al. 1999; Maugeri et al. 2000; Garnier et al. 2007). Evidence was found, that at lower water salinity BRD disease in mussels is much more severe (Reid et al. 2003). This assumption also correlates with our results. In the months of August, September, and October the lowest salinity of the seawater was reported in the region of Arkutino 11.2 ppt, which is also associated with the emergence of transitional species of *Citrobacter farmeri* and pathogenic coliforms (Table 2; Fig. 1). Romalde et al. (2012), found a wide variety of species of *Pseudomonas* sp, which make up about 52.8% of the microbiota in bivalves. These results completely correlate with the results obtained by us and are represented in Table 2. This fact shows that the genus *Pseudomonas* is one of the main groups of microbiota in mussels, although some seasonal variations can be observed, which are related to the

physicochemical parameters of the environment - temperature, pH and salinity. Our results indicate the need for deeper research on the microbiota of mollusks and on the pathogenic potential of marine bacteria, using both culture-dependent and molecular methods. To our knowledge, these are the first data of cultivated bacterial species in mussels from the Black Sea.

Conclusion

When studying the microbiota of populations of different species of bivalves, it is very important to know their sanitary status, as well as to determine the pathobiological basis of periodic outbreaks of diseases affecting these populations. Our results demonstrated the presence of bacterial species of genera *Pseudomonas*, *Enterococcus*, *Escherichia*, *Citrobacter*, and *Acinetobacter* in wedge clams *Donax trunculus* (Linnaeus, 1758). We found that the concentrations of *Escherichia vulneris* exceed 190 times the maximum available values according to Ordinance No. 4/20.10.2000. Inflated concentrations of coliforms in the summer attracted very special attention, indicating a seasonal worsening of the conditions of the seawater as a consequence of anthropogenic activity. We supposed that the pollution was very serious bearing in mind that the habitat of the wedge clams is in depth within the sand. The other important conclusion is the dominance of *Pseudomonas*, found in the mussels, which correspond to some seasonal variations related to the physicochemical parameters of the environment, such as - temperature, pH and salinity. With the worldwide increase in bivalve consumption, we would like to point out the possibility of the emergence of new diseases due to the interaction between the pathogen, the host, and the environment.

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